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Structure of Plant Cell Walls[1]

XII. IDENTIFICATION OF SEVEN DIFFERENTLY LINKED GLYCOSYL RESIDUES ATTACHED TO O-4 OF THE 2,4-LINKED L-RHAMNOSYL RESIDUES OF RHAMNOGALACTURONAN I

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MICHAEL MCNEIL, ALAN G. DARVILL, AND PETER ALBERSHEIM[2]
Department of Chemistry, University of Colorado, Boulder, Colorado 80309

ABSTRACT

Seven differently linked glycosyl residues have been found to be glycosidically linked to O-4 of the branched 2,4-linked L-rhamnosyl residues contained in the rhamnosyl and galacturonosyl backbone of the cell wall pectic polysaccharide rhamnogalacturonan I. These seven glycosyl residues are, therefore, the first residues of at least seven different side chains attached to the rhamnogalacturonan backbone. These first side chain glycosyl residues are 5-linked L-arabinofuranosyl and terminal 3-, 4-, 6-, 2,6-, and 3,6-linked D-galactopyranosyl residues. The existence of at least seven different side chains in rhamnogalacturonan I indicates that rhamnogalacturonan I is either an exceedingly complex polysaccharide or that rhamnogalacturonan I is a family of polysaccharides with similar or identical rhamnogalacturonan backbones substituted with different side chains.

RG-I,[3] a pectic polysaccharide, has been enzymically solubilized and purified from isolated primary cell walls of suspension-cultured sycamore (*Acer pseudoplatanus*) cells (10). The polysaccharide contains a rhamnogalacturonan backbone composed of approximately equal amounts of linear 2-linked and branched 2,4-linked L-rhamnosyl residues interspaced with linear 4-linked D-galacturonosyl residues (10). The 2-linked L-rhamnosyl residues are glycosidically attached to O-4 of D-galacturonosyl residues and have D-galacturonosyl residues glycosidically attached to them at O-2, and the 2,4-linked L-rhamnosyl residues have D-galacturonosyl residues glycosidically attached at O-2 (10).

The glycosyl residues attached at O-4 of the 2,4-linked L-rhamnosyl residues and the glycosyl residues to which the 2,4-linked L-rhamnosyl residues are themselves glycosidically attached have not been established. It has long been proposed that chains of D-galactosyl and L-arabinosyl residues are attached to the O-4 of the 2,4-linked L-rhamnosyl residues (9), but there is no direct evidence establishing this important point. The results described here identify glycosyl residues attached to O-4 of the 2,4-linked L-rhamnosyl residues.

MATERIALS AND METHODS

Isolation of RG-I from Suspension-Cultured Sycamore Cell Walls. RG-I was isolated from suspension-cultured sycamore (*Acer pseudoplatanus*) cell walls as described (10).

***O*-Methylation, Dimethylsulfinyl Anion-Elimination, *O*-Ethylation, and Preparation of Partially *O*-Alkylated Alditol Acetates.** These procedures were performed as described (4, 8, 10).

Oxidation to Keto Groups of the Unsubstituted Hydroxyl Groups Produced by Dimethylsulfinyl Anion-Elimination Ion-Elimination of *O*-Methylated RG-I. The oxidation of unsubstituted hydroxyl groups was performed using the method of Corey and Kim (5) as described by Svensson (13). The oxidizing agent was prepared by the dropwise additon of 0.4 ml of DMSO to 1.2 ml of 1 M chlorine in dichloromethane at −45°C (in a dry ice-acetonitrile bath). The dichloromethane had been dried by passage through a column of aluminum oxide (Woeln neutral activity grade 1). Approximately 4 mg of methylated, dimethylsulfinyl anion-eliminated RG-I, contained in 0.4 ml of DMSO, was added dropwise to the oxidizing agent and the resulting suspension stirred 5 h at 45°C. Two ml of triethylamine was then added and the suspension allowed to warm to room temperature, after which 2 ml of chloroform was added. This solution was extracted four times with 4 ml of 1% HCl. The HCl layers were combined and extracted three times with 2 ml of chloroform. All of the chloroform layers were combined, concentrated, and purified on an LH-20 column as described (10) to yield the *O*-methylated, dimethylsulfinyl anion-eliminated, oxidized RG-I.

Base-Catalyzed Elimination of *O*-Methylated, Dimethylsulfinyl Anion-Eliminated, Oxidized RG-I. The *O*-methylated, dimethylsulfinyl anion-eliminated, oxidized RG-I was subjected to a second base-catalyzed elimination reaction, this time by the milder procedure of Aspinall *et al.* (2). The oxidized material (~1 mg) was dissolved in 200 μl of dry benzene. Fifty μl of the organic base DBU (Aldrich) and 25 μl of acetic anhydride (distilled from sodium acetate) were added and the sealed reaction mixture heated for 24 h at 100°C. After the reaction mixture was cooled, approximately 1 ml of chloroform was added; this chloroform mixture was extracted three times with 0.5 ml of 1% HCl. The partially *O*-methylated, partially *O*-acetylated RG-I oligosaccharides in the chloroform layer were concentrated to dryness.

De-*O*-Acetylation and Reduction of the Partially *O*-Methylated Partially *O*-Acetylated RG-I Oligosaccharides. These reactions were accomplished by a single treatment with 1 M ammonia in ethanol containing 10 mg/ml of sodium borodeuteride (S. Svensson, personal communication). The reaction mixture was incubated for 1 h at room temperature and then for 15 min at 50°C. Excess sodium borodeuteride was decomposed by addition of acetic acid and borate was removed by repeated evaporations with methanol. This procedure yielded a mixture of partially *O*-methylated oligosaccharide alditols. The hydroxyl groups produced by the de-*O*-acetylation and reduction procedures were labeled by a second *O*-methylation step, but this time using trideuteromethyl iodide.

Preparation and Purification of Oligosaccharide 'A'. RGOI was *O*-trideuteromethylated, dimethylsulfinyl anion-eliminated, and *O*-ethylated as described (10). After purification of the reaction

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[2] To whom correspondence should be addressed.

[3] Abbreviations: RG-I, rhamnogalacturonan I; DMSO, dimethylsulfoxide; DBU, 1,5-diazabicyclo[5.4.0]undec-7-ene.

products by LH-20 chromatography, oligosaccharide A was further purified by reverse-phase HPLC using 50% acetonitrile in water with refractive index detection as described (15).

Preparation and Purification of Oligosaccharide Alditols 'B' and 'C'. The D-galacturonosyl residues in RG-I were carboxyl-reduced by the carbodiimide method (10, 14) using sodium borodeuteride in deuterium oxide. The carboxyl-reduced RG-I was *O*-methylated, partially hydrolyzed, reduced and *O*-ethylated using methods described previously (15). The resulting partially *O*-methylated, partially *O*-ethylated oligosaccharide alditols were fractionated and partially characterized by HPLC-MS analysis as described (11). A Hewlett-Packard 108 4B liquid chromatograph coupled to a Hewlett-Packard 5985 mass spectrometer system using a Hewlett-Packard HPLC-MS interface was employed for the HPLC-MS analysis. A DuPont prepacked Zorbax ODS column (4.6 mm i.d. × 25 cm) was used for the reverse-phase HPLC. The flow rate was 1 ml/min and the solvent was a linear gradient of acetonitrile in water programmed from 50 to 65% acetonitrile in 45 min.

Approximately 3% of the HPLC effluent was passed through the HPLC-MS interface for direct MS analysis; the remaining 97% of the effluent was collected in 0.3-ml fractions using a Gilson model FC-80K microfractionator. Chemical ionization mass spectra result from this analysis and are produced by using the acetonitrile/water solvent as the reactant gas (11). Oligosaccharides B and C were located by plotting m/e 498 (the M + 1 ion) *versus* time.

Those HPLC fractions containing oligosaccharides B and C were pooled separately and blown to dryness. These samples were dissolved in chloroform and analyzed by GC-MS (electron impact).

GC-MS. GC-MS of partially *O*-alkylated alditol acetates was performed as described (4, 10). GC-MS (electron impact) of oligosaccharides A, B, and C was done on a Hewlett-Packard 5985 GC-MS system using a SE30 (LKB, Broma, Sweden) open tubular glass capillary column using splitless injection. The gas chromatograph was programmed with an initial temperature of 120°C for 2 min followed by a temperature rise at 30°/min to 200°C, followed by another temperature rise of 8°/min to 300°C. GC-MS (chemical ionization) of oligosaccharide A was done on the same Hewlett-Packard 5985 GC-MS system using ammonia as a reactant gas at a source pressure of 0.4 torr and a source temperature of 150°C.

LH-60 Chromatography. LH-60 gel filtration chromatography of both carboxyl-reduced *O*-methylated RG-I and *O*-methylated RG-I in which the carboxyl groups were not reduced were performed on a 1.7 × 50 cm column of LH-60 (Pharmacia) equilibrated in chloroform:methanol (1:1). Fractions were collected and analyzed colorimetrically as described for LH-20 gel filtration chromatography (10).

RESULTS

Identification, by a Series of Elimination and Oxidation Reactions, of the Glycosyl Residues Attached to O-4 of the 2,4-Linked Rhamnosyl Residues. It has been shown previously that methylation of RG-I followed by treatment with 0.9 M dimethylsulfinyl anion (base-catalyzed uronosyl elimination conditions [3, 8]) results in conversion of the 2,4-linked L-rhamnosyl residues into derivatives of 4-linked L-rhamnosyl residues. The 4-linked L-rhamnosyl derivatives possess an *O*-methyl group at C-3 and an unsubstituted hydroxyl at C-2 (10). The glycosyl residues attached at O-4 of the 4-linked L-rhamnosyl derivatives were not identified. The 4-linked L-rhamnosyl residues were themselves glycosidically linked to an unidentified component. We present evidence below (see discussion of disaccharide A) that the 4-linked L-rhamnosyl residues are in fact methyl glycosides.

The mechanism by which the L-rhamnosyl methyl glycosides were formed has not been investigated. An understanding of how this reaction occurs was not required in order to carry out the experiments described in this paper. What was essential for this investigation is that the 2,4-linked L-rhamnosyl residues of RG-I are converted by *O*-methylation and dimethylsulfinyl anion-elimination to 4-linked L-rhamnosyl residues containing an unsubstituted hydroxyl on C-2 (10). The production of these rhamnosyl residues allowed reactions to be carried out to identify the glycosyl residues attached to O-4 of the L-rhamnosyl residues which were 2,4-linked in RG-I.

The series of reactions, summarized in Figure 1, were undertaken to identify the glycosyl residues attached to O-4 of the 4-linked L-rhamnosyl derivatives produced by dimethylsulfinyl anion-elimination of the *O*-methylated RG-1. In reaction 1, the free hydroxyl group at C-2 of the 4-linked L-rhamnosyl derivatives was oxidized to a keto group (see "Materials and Methods"). The percent of molecules in which the hydroxyl group at C-2 was oxidized was estimated by hydrolyzing, reducing, and *O*-acetylat-

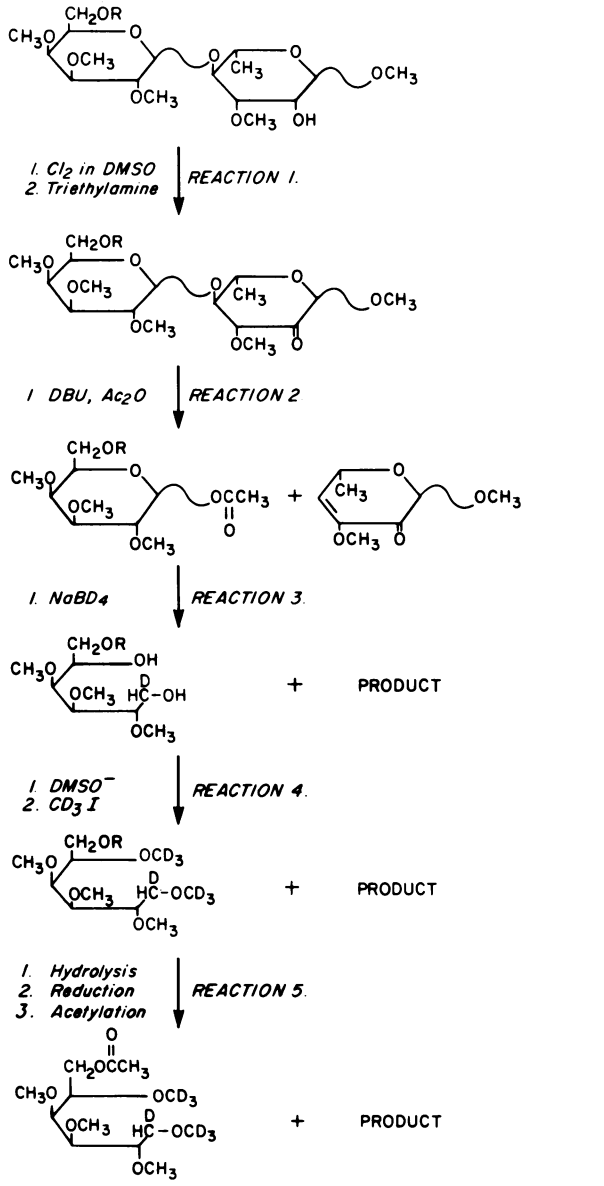

FIG. 1. Reactions used to identify the glycosyl residues attached to O-4 of the 4-linked rhamnosyl derivatives formed by dimethylsulfinyl anion-elimination of RG-I. The reaction sequence is illustrated with a 6-linked galactosyl residue attached to O-4 of the rhamnosyl derivative.

ing a small aliquot of the oxidized oligosaccharide. The unoxidized 4-linked derivative yields 1,2,4,5-tetra-*O*-acetyl-3-*O*-methyl rhamnitol while the 2-keto derivative does not give this compound. Over 85% of the 3-*O*-methyl alditol acetate derived from the 4-linked L-rhamnosyl derivative was lost by the oxidation procedure.

The newly formed keto group at C-2 allows base-catalyzed elimination of the glycosyl residues attached to O-4 of the 4-linked L-rhamnosyl derivatives. If dimethylsulfinyl anion were to be used to catalyze this elimination, the glycose residues eliminated from O-4 would be destroyed by a second elimination reaction (8). Therefore, a different elimination procedure, designed to protect glycoses from elimination, was used (2). This reaction is catalyzed by the organic base DBU. Acetic anhydride, present during the elimination, rapidly *O*-acetylates the C-1 hydroxyl groups of the ring forms of the released glycoses (reaction 2), thus protecting the released glycoses from base-catalyzed elimination. At the same time, the glycosyl residues which were attached to O-4 of the L-rhamnosyl derivatives are uniquely labeled by the *O*-acetyl groups on C-1.

The next series of reactions, summarized in Figure 1, converted the glycosyl residues *O*-acetylated at O-1 to specifically labeled partially *O*-methylated alditol acetates. The glycosyl residues released from O-4 of the L-rhamnosyl derivatives were de-*O*-acetylated and reduced to alditols in a single step (reaction 3), producing unsubstituted free hydroxyls on C-1 and C-5 (for former pyranosyl residues) or on C-1 and C-4 (for former furanosyl residues). The unsubstituted hydroxyls were then *O*-trideuteromethylated (reaction 4). Thus, the glycosyl residues originally attached to O-4 of the 2,4-linked L-rhamnosyl residures were labeled with trideuteromethyl groups on O-1 and O-5 or on O-1 or O-4. These derivatized residues were then subjected to hydrolysis, $NaBD_4$ reduction, and *O*-acetylation to form the corresponding partially *O*-methylated, partially *O*-trideuteromethylated alditol acetates which were identified by GC-MS (reaction 5).

We realized that some glycosyl residues of RG-I might be reduced and consequently trideuteromethylated at O-1 and O-5 (or O-4) by a mechanism other than the intended one outlined in Figure 1. If this occurred, such residues would incorrectly appear to be attached to the O-4 position of the 2,4-linked L-rhamnosyl residues. To correct for this possibility, a control experiment was performed in which RG-1 was treated exactly as outlined in Figure 1 except that the oxidation step (reaction 1) was omitted. This control should detect glycosyl residues labeled on O-1 and O-5 or O-4 by other than the desired reactions.

The per-*O*-alkylated alditol acetates with *O*-trideuteromethyl groups on C-1 and C-5 (or C-4) detected after hydrolysis, reduction, and *O*-acetylation of the test sample of RG-I (*O*-methylated, treated with dimethylsulfinyl anion, oxidized, eliminated with DBU in the presence of acetic anhydride, de-*O*-acetylated, reduced, and *O*-trideuteromethylated) and of the control sample of RG-I (treated the same way except for the omission of the oxidation step) are listed in Table I. Seven differently linked glycosyl residues were found to be attached in RG-I to O-4 of the 2,4-linked L-rhamnosyl residues: 5-linked L-arabinofuranosyl and terminal, 3-, 4-, 6-, 3,6-, and 2,6-linked D-galactopyranosyl residues. The sum of the mol % of these glycosyl residues (4.5%) is 75% (see footnote 'd' of Table I) of the mol % of rhamnosyl residues (6.0 mol %) known to possess glycosyl residues attached to O-4 (10). The control sample yielded less than 15% of each of the galactosyl derivatives produced with the oxidized sample and less than 25% of the arabinosyl derivative produced with the oxidized sample. The reason the derivatives were detected in the control experiments is not known.

Isolation of Oligosaccharides from RG-I. Oligosaccharide A (Fig. 2), which confirms that terminal D-galactosyl residues are attached to O-4 of some of the 2,4-linked L-rhamnosyl residues, was isolated after *O*-trideuteromethylation and dimethylsulfinyl anion-elimination of RG-I, followed by *O*-ethylation and HPLC of the resulting fragments. Oligosaccharide A was separated into two fractions, each of which gave identical mass spectral data. Presumably, they are the α- and β-trideuteromethyl glycosides (see below for explanation).

The structure of oligosaccharide A was established as follows. (a) Hydrolysis of an aliquot of each of the two HPLC peaks containing oligosaccharide A followed by reduction and *O*-acetylation yielded, from each peak, equal molar amounts of 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-trideuteromethyl D-galactitol and 1,4,5-tri-*O*-acetyl 3-*O*-trideuteromethyl 2-*O*-ethyl L-rhamnitol. (b) Chemical ionization (ammonia) GC-MS analysis of the two oligosaccharide A HPLC peaks gave mass spectra containing an M+ 18 ion of m/e 474 which corresponds to the expected mol wt, 456, of the disaccharide trideuteromethyl glycoside. (c) Electron

Table I. *Partially O-Methylated, Partially O-Trideuteromethylated Alditol Acetates Obtained after DBU Elimination of Oxidized and of Nonoxidized Samples of Derivatized RG-I*

The RG-I had been previously *O*-methylated and uronosyl-eliminated.

Glycosyl Residue	Position of *O*-Methyl Groups	Position of *O*-Trideutero-Methyl Groups	Deduced Linkage[a]	Oxidized[b]	Nonoxidized[c]
				mol %[d]	
Arabinosyl	2,3	1,4	5	1.8	0.4
Galactosyl	2,3,4,6	1,5	terminal	—[e]	0
	2,4,6	1,5	3	0.9	0.05
	2,3,6	1,5	4	0.9	0.1
	2,3,4	1,5	6	0.4	0.02
	2,4	1,5	3,6	0.4	0.05
	3,4	1,5	2,6	0.1	0

[a] The deduced linkage was determined by the positions of the *O*-acetyl groups.

[b] Average of three experiments.

[c] Average of two experiments.

[d] The mol % reported is the percent of all the partially *O*-alkylated alditol acetates recovered. This includes all the *O*-methylated alditol acetates with no *O*-trideuteromethyl groups at C-1 and C-5 (or C-4) (residues in RG-I that are not directly attached to O-4 of the 2,4-linked L-rhamnosyl residues). The mol % of 2,4-linked L-rhamnosyl residues in unmodified RG-I is 6%. The sum of the residues attached to O-4 of the 2,4-linked L-rhamnosyl residues recovered after oxidation of RG-I is 4.5% (not including the volatile 2,3,4,6-tetra-*O*-methyl 1,5-di-*O*-trideuteromethyl derivative of terminal galactosyl residues) which is in good agreement with the expected total of 6%.

[e] Present but not quantitated due to the volatility of this derivative.

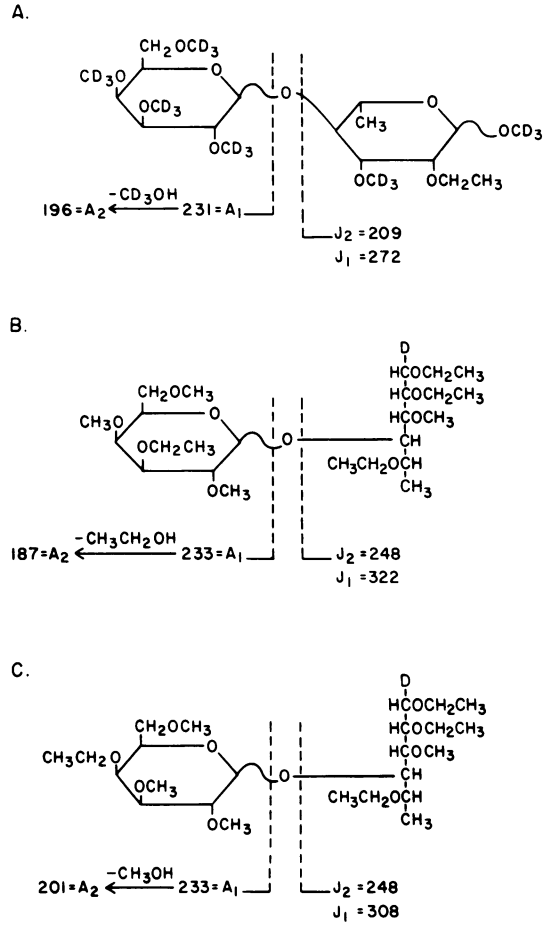

FIG. 2. Structures of peralkylated oligosaccharides A, B, and C. The formation of some of the diagnostic ions present in their electron impact mass spectra are illustrated. The J_1 ion is formed by a complicated process which is not illustrated in the figure but is explained in References 7 and 11.

impact GC-MS analysis (Fig. 2) gave a mass spectrum which contained the following ions (using the nomenclature proposed by Kochetkov and Chizhov [7]): m/e 272 (rhamnose trideuteromethyl glycoside J_1 [formation not illustrated; see Ref. 4]), m/e 209 (rhamnose trideuteromethyl glycoside J_2), m/e 231 (galactose A_1), and m/e 196 (galactose A_2). These results establish that oligosaccharide A originated as a disaccharide fragment in which a terminal galactosyl residue is attached to 0-4 of a rhamnosyl residue; the rhamnosyl residue was 2,4-linked in RG-1. The *O*-ethyl group at C-2 of the L-rhamnosyl residue of disaccharide A is explained by the free hydroxyl group on this carbon at the time of *O*-ethylation. The free hydroxyl was formed during the base elimination procedure by cleavage of the D-galacturonosyl residue that was attached to O-2 of the rhamnosyl residue in intact RG-I (3, 10).

The finding of an *O*-trideuteromethyl group at C-1 of the L-rhamnosyl residue of disaccharide A was unexpected. We have investigated how this trideuteromethyl rhamnoside is formed and conclude that the glycosidic linkages between 2,4-linked L-rhamnosyl residues and O-4 of D-galacturonosyl residues are cleaved by the dimethylsulfinyl anion base treatment that was used during the original *O*-trideuteromethylation of RG-I and prior to addition of trideuteromethyl iodide. When trideuteromethyl iodide was added, a trideuteromethyl group was then attached at O-1 of the 2,4-linked L-rhamnosyl residues yielding the α- and β-trideuteromethyl L-rhamnosides. One remarkable characteristic of this unexpected reaction is that it occurs to over 90% of the 2,4-linked L-rhamnosyl residues of RG-I but apparently to less than 10% of the 2-linked L-rhamnozyl residues of RG-I. This is true even though D-galacturonosyl residues are attached to O-2 of both the 2- and 2,4-linked rhamnosyl residues and even though both rhamnosyl residues are glycosidically linked to O-4 of D-galacturonosyl residues (10). This explanation for the presence of the *O*-trideuteromethyl group at C-1 of only the 2,4-linked L-rhamnosyl residues is supported by the following observations.

(a) The formation of the trideuteromethyl glycosides of the 2,4-linked L-rhamnosyl residues and the apparent failure to form the trideuteromethyl glycosides of the 2-linked L-rhamnosyl residues was established in experiments in which *O*-methylated RG-I was treated with dimethylsulfinyl anion to cause β-elimination of the *O*-methylated uronosyl residues. It has been demonstrated previously that the glycosyl residues released from O-4 of uronosyl residues during dimethylsulfinyl anion-degradation of *O*-methylated uronosyl residues will themselves be degraded (2, 8). However, methyl glycosides would not be degraded as the methyl glycosides do not contain a free aldehyde function at C-1 (2, 8). Dimethylsulfinyl anion-catalyzed degradation of *O*-methylated RG-I results in the loss of 90% of the 2-linked L-rhamnosyl residues (presumably still glycosidically linked to O-4 of D-galacturonosyl residues after the original *O*-methylation reaction) but degrades none of the 2,4-linked L-rhamnosyl residues (presumably released from O-4 of D-galacturonosyl residues and methylated at O-1 during the original *O*-methylation reaction) (10).

(b) LH-60 gel filtration column chromatography of RG-I which had been de-esterified, carboxyl-reduced, and *O*-methylated yields a single peak which elutes in the void volume of the column, whereas, RG-I which was de-esterified and *O*-methylated without carboxyl-reduction elutes as a very broad peak with most of the material in the partially included volume of the column. This observation shows that a single methylation reaction has altered the sizing properties of native RG-I in which the carboxyl groups had not been reduced. We presume that the observed reduction in the mol wt of native RG-I results from base-catalyzed elimination of D-galacturonosyl residues in the rhamnogalacturonan backbone (and formation of the methyl glycoside of the 2,4-linked L-rhamnosyl residues).

(c) An analogous formation of methyl glycosides during base-catalyzed elimination of two other polysaccharides has recently been demonstrated in this laboratory (L.-E. Franzén, P. Åman, A. G. Darvill, M. McNeil, and P. Albersheim, unpublished results). The acidic polysaccharides secreted by *Rhizobium trifolii* and *Rhizobium phaseoli* both contain a branched 4,6-linked D-glucosyl residue glycosidically attached to O-4 of a D-glucuronosyl residue. After a single *O*-methylation, approximately 50% of the branched D-glucosyl residue was shown to be present as a mixture of its α and β methyl glycosides.

(d) All or almost all of the 2,4-linked L-rhamnosyl residues of RG-I are attached to O-4 of D-galacturonosyl residues. Therefore, the branched rhamnosyl residues are in a position analogous to that of the branched glucosyl residues of the rhizobium polysaccharides, residues which were shown to form methyl glycosides during the *O*-methylation reaction. The attachment of the branched rhamnosyl residues to O-4 of galacturonosyl residues was established by analysis of the per-*O*-alkylated oligosaccharide alditols resulting from partial hydrolysis, reduction and ethylation of carboxyl-reduced *O*-methylated RG-I (11, 14). The analysis yields several oligosaccharide alditols in which 2,4-linked L-rhamnosyl residues are attached glycosidically to O-4 of D-galacturonosyl residues (M. McNeil, A. G. Darvill, P. Albersheim, unpublished results). In the same analysis, no peralkylated oligosaccharides were detected in which 2,4-linked L-rhamnosyl residues were glycosidically attached to any other glycosyl residue or to any position other than to O-4 of D-galacturonosyl residues.

The rationale we have presented to explain the *O*-trideutero-

methyl group at C-1 of oligosaccharide A must remain tentative, as the mechanism of the reaction is not understood and no explanation can be offered as to why the reaction occurs with the 2,4-linked but apparently not with the 2-linked L-rhamnosyl residues. The possibility that the D-galacturonosyl residues to which the 2,4-linked L-rhamnosyl residues are glycosidically attached eliminate because those galacturonosyl residues possess endogenous methyl esters is not correct because base-catalyzed de-esterification followed by *O*-methylation and elimination of RG-I still results in the formation of the methyl glycosides of the 2,4-linked L-rhamnosyl residues. The isolation of this particular disaccharide derivative, regardless of the mechanism by which the trideuteromethyl glycoside of the L-rhamnosyl residue is formed, confirms that, in RG-I, terminal D-galactosyl residues are attached to O-4 of some of the 2,4-linked L-rhamnosyl residues.

Peralkylated disaccharide alditols B and C (Fig. 2) were isolated from RG-I by HPLC after carboxyl-reduction, *O*-methylation, partial hydrolysis, reduction, and *O*-ethylation using procedures described (11, 15). The resulting per-*O*-alkylated oligosaccharide alditols were analyzed by HPLC mass spectrometry as described in "Materials and Methods." Per-*O*-alkylated disaccharide alditols B and C were identified by their chemical ionization mass spectra obtained by HPLC-MS (11), by their electron impact mass spectra (7) obtained by GC-MS (Fig. 2; and "Materials and Methods"), and by identification of the partially *O*-alkylated alditol acetates derived from B and C after hydrolysis, reduction with $NaBD_4$, and *O*-acetylation (4). The details of these three methods of analysis have recently been reviewed (11).

The presence of *O*-ethyl groups at C-3 and C-4 of the galactosyl residues in per-*O*-alkylated disaccharide alditols B and C, respectively, indicate points of attachment of other glycosyl residues in the original polysaccharide. Thus, the galactosyl residue in B was originally 3-linked and in C was originally 4-linked (15). The *O*-ethyl groups on C-2 of the rhamnosyl residues of B and C show that, in both cases, the rhamnosyl residues were originally 2,4-linked. Thus, oligosaccharides B and C confirm the attachment of 3-linked and 4-linked D-galactosyl residues to O-4 of some of the 2,4-linked L-rhamnosyl residues of RG-I.

DISCUSSION

The O-4 position of 2,4-linked L-rhamnosyl residues has long been proposed as the position of attachment of glycosyl side chains to the pectic polymers in plant cell walls (9). The work presented in this paper confirms this structural feature and establishes that at least seven different side chains are attached to O-4 of the 2,4-linked L-rhamnosyl residue. The first glycosyl residues of the seven different side chains are illustrated in Figure 3. As only the first residue in the side chains has been identified, the number of structurally unique side chains could be much larger.

The sequence of reactions used to determine the seven differently linked glycosyl residues attached to O-4 of the 2,4-linked L-rhamnosyl residues are complex. Several observations confirm that the results are valid and not artifacts of the procedures used. (a) In control samples, when a single critical reaction was omitted from the analysis, the yields of the derivatives which established the nature of the differently linked glycosyl residues attached to O-4 of the rhamnosyl residues were reduced, on average, by 90% (see Table I). (b) The sequence of reactions used to identify the glycosyl residues attached to O-4 of rhamnosyl residues was carried out on three separate occasions with similar results each time. (c) the mol % of the total glycosyl residues in RG-I (see Table I) which are accounted for by the seven glycosyl residues found attached to O-4 of the rhamnosyl residues is approximately 4.5%. This agrees well with the observation that 2,4-linked L-rhamnosyl residues account for 6% of the glycosyl residues of RG-I (10). (d) Three-linked D-galactosyl residues are present in relatively small amounts (1.8 mol %) in RG-I (10), but half of them (0.9 mol %; see Table I) are attached at O-4 of 2,4-linked L-rhamnosyl residues. This would not be expected if randomization errors were present in the analysis. (e) All of the differently linked glycosyl residues found to be attached to O-4 of the L-rhamnosyl residues were previously shown to be part of RG-I (10). In other words, no unexpectedly linked glycosyl residues were found which might have resulted from incomplete reactions or randomization errors. (f) Finally, three of the quantitatively major glycosyl residues attached to O-4 of L-rhamnosyl residues were confirmed by a completely independent method based on isolation of fragments of RG-I.

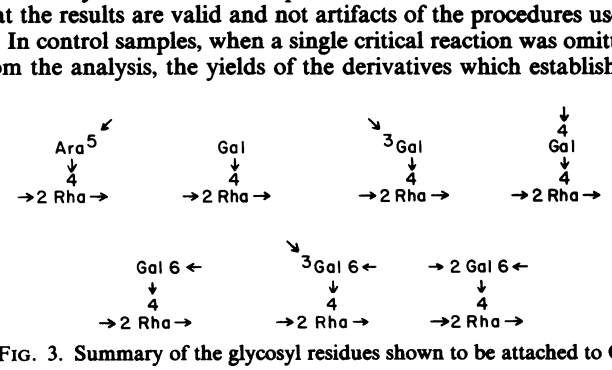

FIG. 3. Summary of the glycosyl residues shown to be attached to O-4 of 2,4-linked L-rhamnosyl residues of RG-I.

The presence of so many differently linked glycosyl residues attached to O-4 of the 2,4-linked L-rhamnosyl residues in RG-I is consistent with recent results which have established the tremendous complexity of the pectic polymers of plant cell walls (9, 10). These data also raise the possibility that RG-I is a family of polysaccharides, all with the same or similar backbones but possessing different side chains. It is possible that in differentiated plant tissues different cells synthesize different RG-I's or varying proportions of different RG-I's.

Plant cell wall polysaccharides or parts thereof have been shown recently to function as regulatory molecules as well as structural polymers (1, 6, 12). The complex structures associated with RG-I suggest fragments of RG-I contain enough information to function as regulatory molecules. Indeed, considering the structural diversity of its side chains, it is difficult to conceive of a purely structural role for RG-I.

LITERATURE CITED

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